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Los Alamos NATIONAL LABORATORY

Construction, characterization and crystal structure of a fluorescent single-chain Fv chimera

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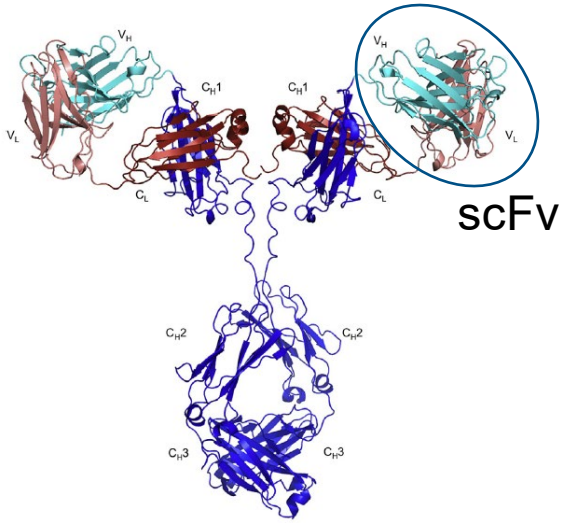
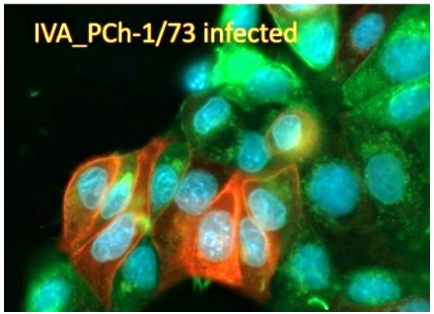
Fluorescent Antibodies: Workhorse of Biomedical Research

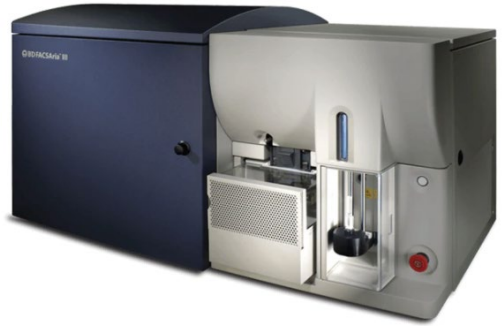
- Antibodies (IgG) are large Y shaped proteins of our immune system that identify and neutralize pathogenic bacteria and viruses
- Monoclonal antibodies are effective therapeutic agents as well as a highly valuable and ubiquitous biomedical research reagent
- Fluorescently labeled antibodies are extensively used in diagnostic and research methods such as immunofluorescent microscopy and flow cytometry.

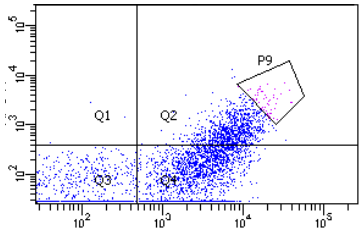


R. Rouet et al. / FEBS Letters 588 (2014) 269–277



N. VELAPPAN ET AL MABS 2020 e1843754-2.





Generating Fluorescent Antibodies: Current techniques and Concerns

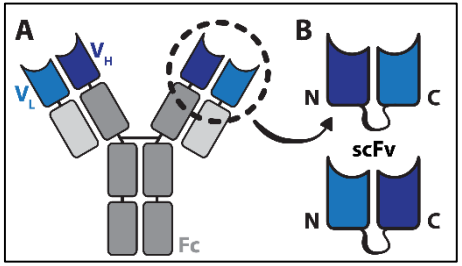
- Fluorescent IgG are typically produced by chemical reaction, e.g. a succinimidyl ester functional group attached to a fluorophore core reacts with primary amines to label the antibody.

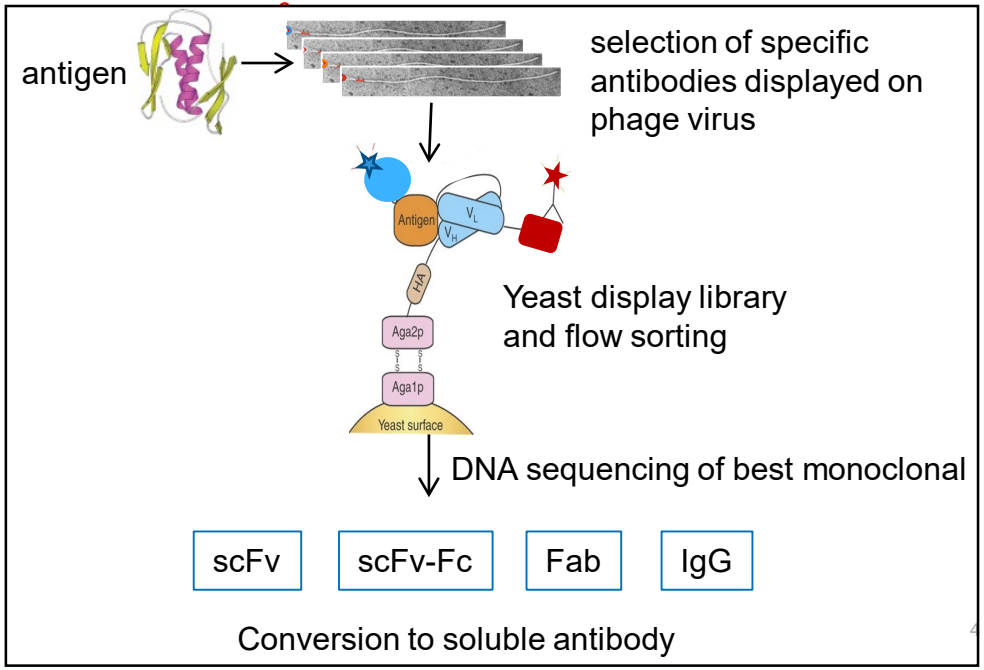
Potential Problems

- Labeling of primary amines in the binding region may block antigen recognition
- Different antibodies react with different fluorophores at different rates, creating variability in experimental settings
- Labeling reaction is stochastic, some antibodies will have large numbers of fluorophores and some very few
- Over conjugation can result in quenching
- Batch-to-batch variations may result in variations in antigen recognition signal

In vitro Generation of Highly Specific Antibodies

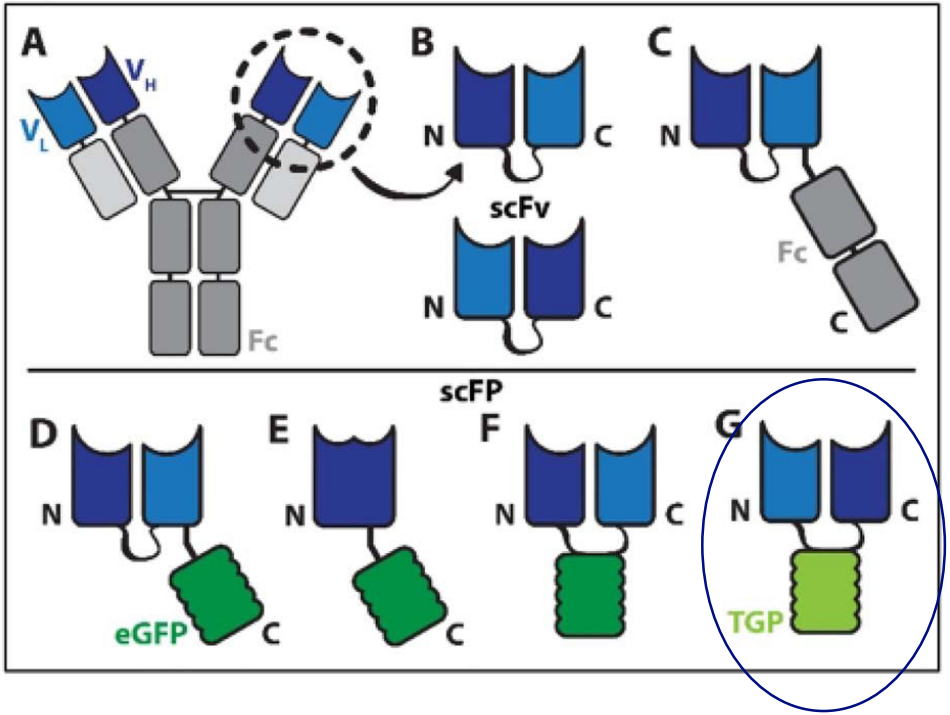
- *In vitro* antibody selection utilizing large combinatorial libraries displayed phage and/or yeast can generate high specific antibodies with high affinity
- These display technologies often use scFv -VH and VL regions with an unstructured linker peptide
- However, scFvs tend to
  - aggregate
  - low expression levels
  - unstable during long-term storage
  - Binding signal detection requires a labeled secondary antibody that recognize an expression tag





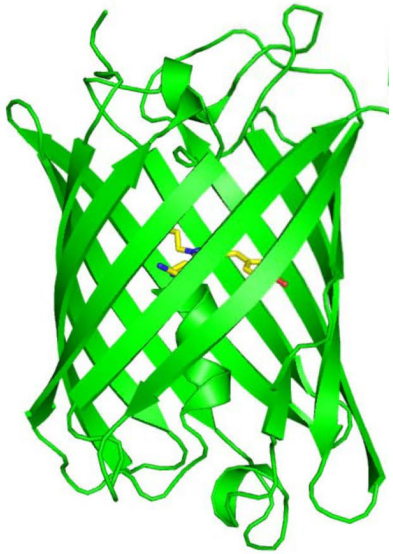
Strategies to improve scFv functionality

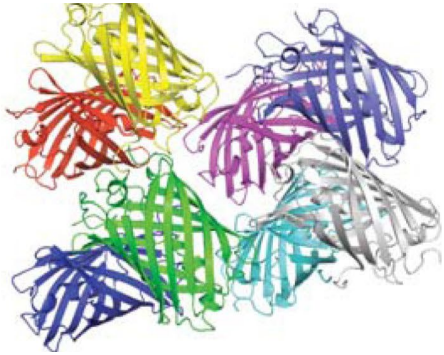
- scFv-Fc improved stability and allow recognition by secondary antibodies
- scFv-Enzyme (e.g. alkaline phosphatase)
- Single chain-fluorescent protein (scFP) chimera
  - FP expression on C-terminus
  - FP protein as linker between VH and VL regions of the scFv



Thermal Green Protein (TGP) based scFPs

- Extremely stable
- Highly soluble
- Non-aggregating green fluorescent protein
- TGP is monomeric
- TGP was engineered by disrupting crystal lattice contacts and introducing high-entropy glutamate residues to improve crystallization and prevent oligomerization.
- Potentially suitable for insertion into scFv genes, substituting the linker originally used to tether the VL and VH.

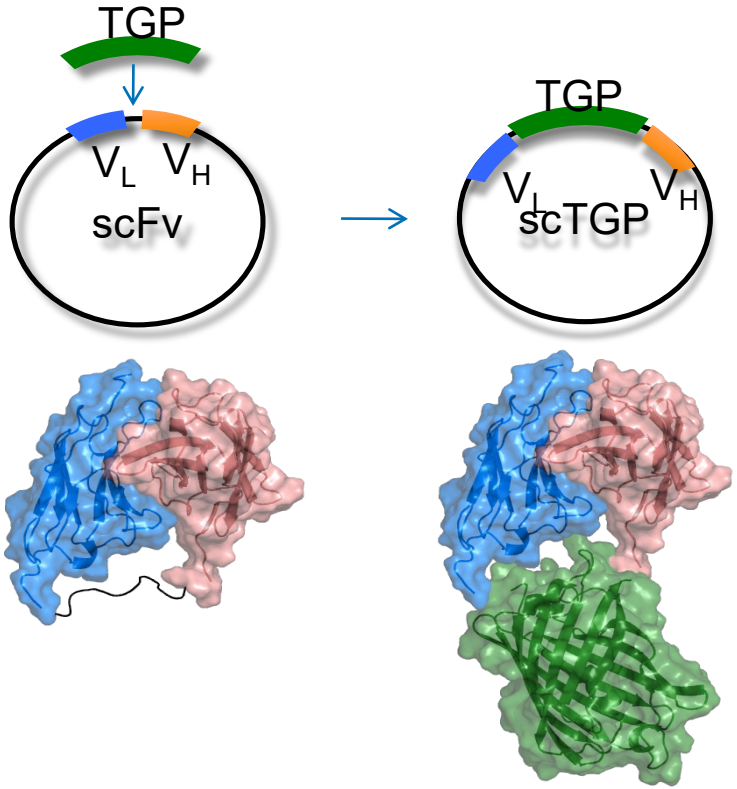




Close et al. Proteins. 2015 83(7): 1225–1237.

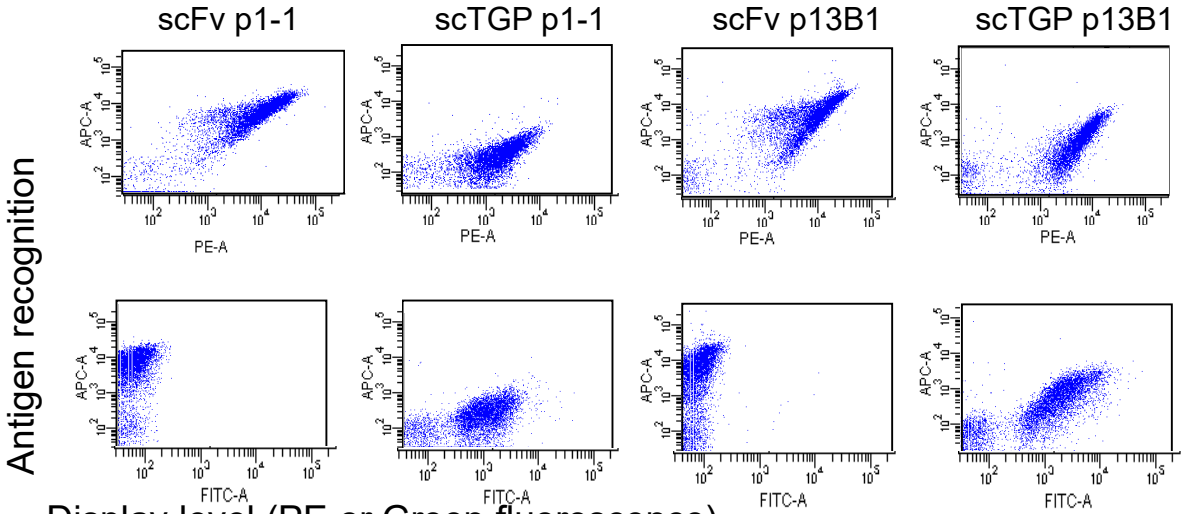
Construction of scTGPs

- Inverse PCR amplified scFv gene and vector sequence
- TGP was amplified with overlapping primers
- Circular polymerase extension cloning (CPEC) assembly was used to create scTGP constricts
  - Yeast display vectors
  - Protein expression vectors



Evaluating scTGP functionality using yeast display

scFvs p1-1 and p13B1 recognizes phospho-tyorosines on the FcεR1 receptor

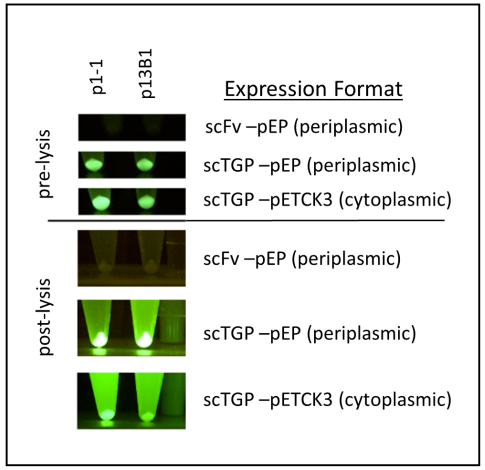


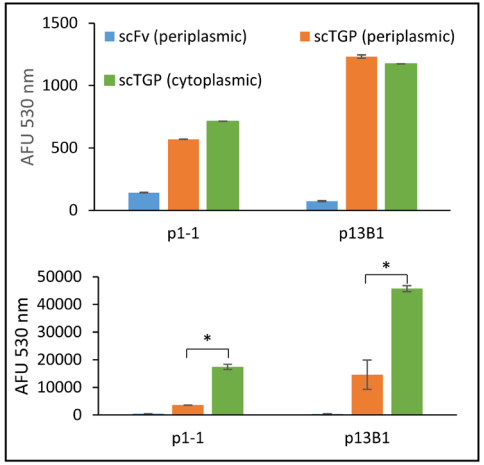
- Yeast can display folded scTGP
- Display level and antigen recognition signal for scTGPs is lower than scFv
- ScTGPs recognize specific antigen with similar affinity (~3x range)

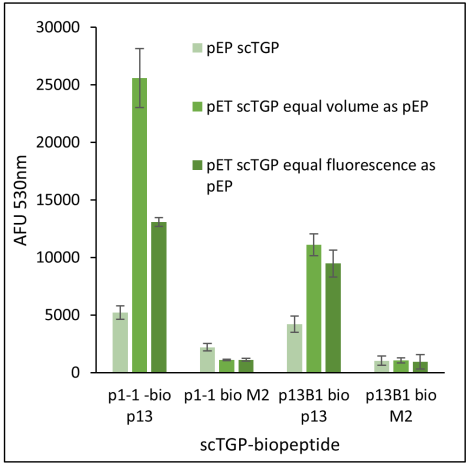
	scFv p1-1	scTGP p1-1	scFv p13B1	scTGP p13B1
Display level (MFI) (SV5-PE)	9111	1280	10,215	5168
Recognition signal for specific target (MFI) (biotinylated target-streptavidin A633)	4696	283	4882	893
Recognition signal for non-specific target M2 (MFI) (biotinylated target-streptavidin A633)	160	127	160	118
K_D values for specific antigen (nM)	2.43±0.22	8.55±2.33	1.40±0.65	0.51±0.56

Determining protein expression system for scTGPs

- scFvs require periplasmic expression
- Fluorescent proteins express in cytoplasm, which usually produces higher quantities of protein
- Evaluated scTGP expression in both periplasm and cytoplasm and compared functionality using FLISA
- Cytoplasmic expression (pET CK3) produced statistically higher amounts of scTGPs and they also recognized the specific antigen in FLISA

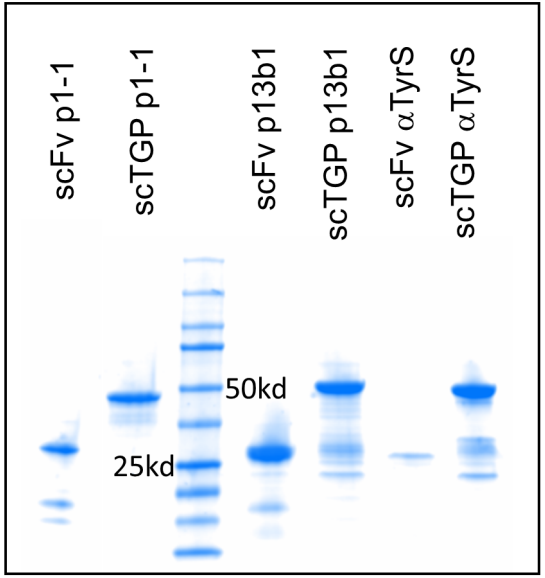






scFv- scTGP Protein Expression Comparison

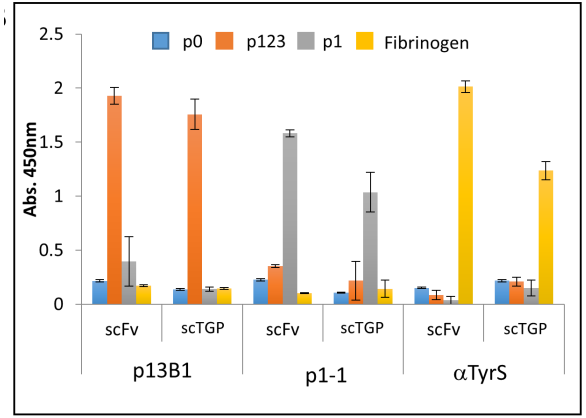
- Three scFv and scTGP pairs were compared for protein yield
- scFvs with good expression levels (p1-1 and p13B1), had comparable expression and yield as scTGPs
- For αTyrS antibody a problem scFv, conversion to scTGP increased purified protein yield by 10x
- Monitoring protein expression and purification is considerably easier for scFPs

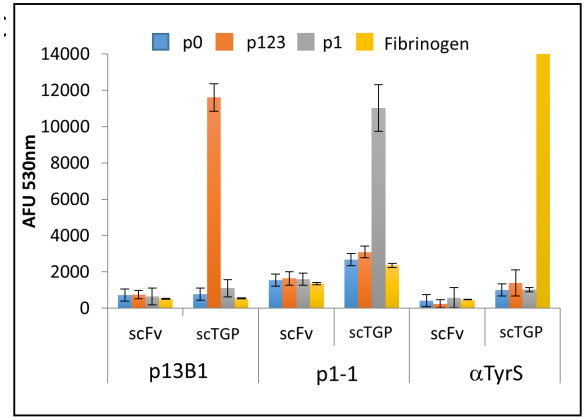


Antibody name	scFv (mg)	scTGP (mg)
p1-1	4.3	6.9
p13B1	5.5	6.9
αTyrS	0.7	6.0

scFv – scTGP Protein Functionality Comparison

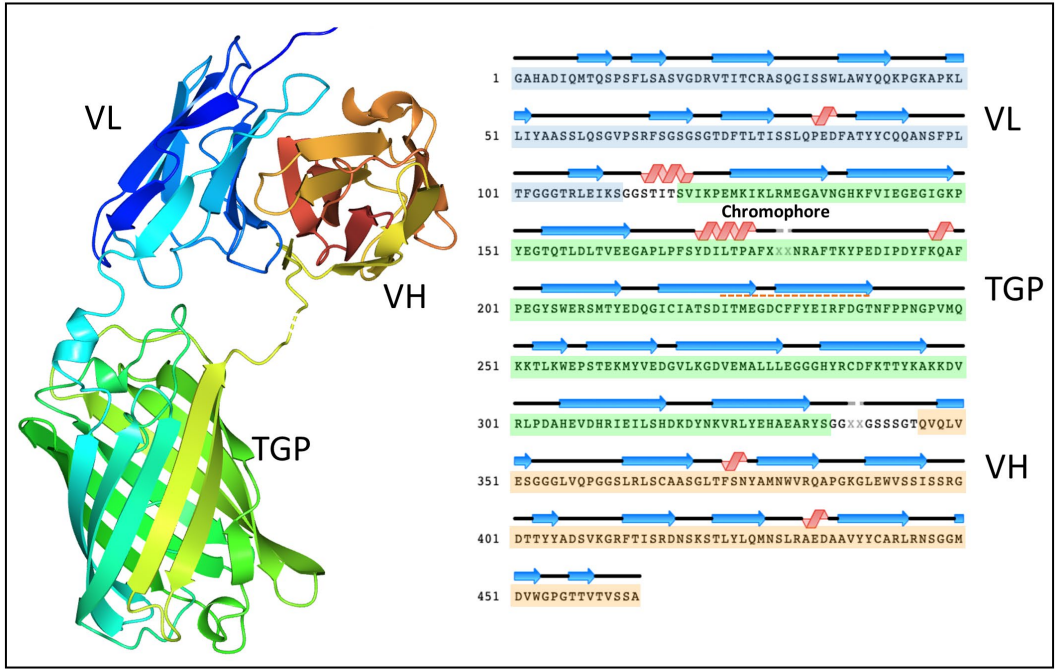
- Equal concentration of scFv and scTGP used to access antigen recognition of three antibodies
- ELISA data showed both antibody formats have equivalent functionality for antigen recognition
- FLISA data shows that antigen recognition by scTGPs can be measured using fluorescence in a single step assay format
- Additional experiments also showed that at equimolar concentrations both antibody format give equivalent binding signal





Crystal Structure of scTGP p1-1

- Bipyramid shaped crystals of scTGP p1-1 formed with 1.6 M citric acid pH 6.2 as crystallization buffer after 2 months.
- Diffracted to better than 2.5 A resolution
- The final *R*work and *R*free values were 16.3% and 21.1%
- The refined structure contains 1 scTGP p1-1, 2 glycerols, and 136 water molecules.



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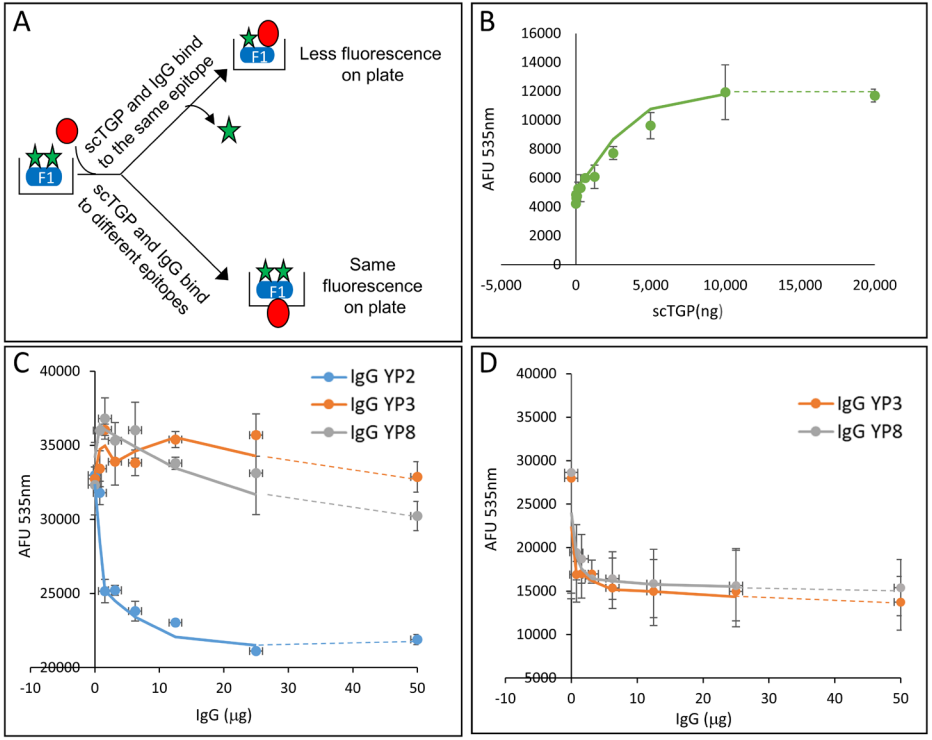
Epitope binning of α*Yersinia pestis* antibodies

- The effect of unlabeled IgGs on scTGPs binding was used to determine whether the two antibody formats bind to same or different epitopes, in a one-step assay (A)
- An scTGP-F1V binding curve (B) was used to estimate the half-saturating concentration of scTGP, optimal for detection of scTGP displacement
- Binding of scTGP was detected by fluorescence in the presence of non-fluorescent IgGs
- Panel C shows that IgG YP3 and YP8 does not displace scTGP 2 (no drop in signal) and IgG YP2 displaces scTGP2 as expected.
- Panel D shows antibodies YP3 and YP8 compete for the same epitope



Conclusions

- The scTGPs are recombinant intrinsically fluorescent antibodies, ready for use in traditional fluorescent antibody based assays
- The scFPs can be constructed as vL-FP-vH format, TGP an extremely stable monomeric fluorescent protein is suitable FP
- The scFPs can be effectively displayed on yeast, where they function similarly to their corresponding scFv counterparts, recognize specific antigen similar affinity
- Functional expression levels of scFPs in the bacterial cytoplasm are relatively high and can rescue some scFvs otherwise expressed at low levels in the periplasm
- Straightforward assessment of expression levels and monitoring of protein purification steps. Antigen recognition in one step binding assays, novel assays such as epitope binning
- TGP is a monomeric fluorescent protein amenable to crystallization. These features provide unique and valuable characteristics to scTGP molecules for their use in protein chemistry
- We present unique antibody format with wide variety of use in research and clinical setting

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